FORM PTO-1390 (REV 10/95)

U. S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: HUBR-1190 (10104965)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): 09/869027

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP99/10378	23 December 1999	23 December 1998

TITLE OF INVENTION
PENICILLINS AS PHARMACEUTICALS FOR THE DOWNREGULATION OF IFNγ PRODUCTION

APPLICANT(S) FOR DO/EO/US
Elisabetta PADOVAN, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ■ This is the FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. □ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ■ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. ■ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ■ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. □ is transmitted herewith (required only if not transmitted by the International Bureau.)
   b. ■ has been transmitted by the International Bureau.
   c. □ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ■ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ■ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. □ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. □ have been transmitted by the International Bureau.
   c. □ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ■ have not been made and will not be made.
8. □ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ■ An unsigned oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ■ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. □ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. □ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ■ A FIRST preliminary amendment.
    □ A SECOND or SUBSEQUENT preliminary amendment.
14. □ A substitute specification.
15. □ A change of power of attorney and/or address letter.
16. □ Other items or information:
17. ■ The follow fees are submitted: $533.00
18. □ Other:

BASIC NATIONAL FEE (37 CFR 1.492(A)(1) - (5)): Search Report has been prepared by the EPO or JPO ..... $860.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... $690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ... $710.00 Neither International preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO .... $1000.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				$860.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24	4	x $9.00	$ 36.00	
Independent	4	1	x $40.00	$ 40.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $250.00	$	
	TOTAL OF ABOVE CALCULATIONS =			$1,066.00	
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$533.00	
SUBTOTAL =				$533.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$0	
TOTAL NATIONAL FEE =				$533.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$0	
TOTAL FEES ENCLOSED =				$533.00	
				Amount to be: refunded	$
				charged	$

a. ■ A check in the amount of $533.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 50-0624 in the amount of $____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ■ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 50-0624. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Norman D. Hanson
FULBRIGHT & JAWORSKI L.L.P.
666 Fifth Avenue
New York, NY 10103
Customer No. 24972

SIGNATURE

Narman D. Hanson June 22, 2001
NAME

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Fulbright & Jaworski L.L.P.
666 Fifth Avenue
New York, New York 10103

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(Name of Depositor)

(Signature of Depositor)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Elisabetta PADOVAN et al.
International Application No.	:	Based on PCT Application No. PCT/EP99/10378
International Filing Date	:	HEREWITH
For	:	PENICILLINS AS PHARMACEUTICALS FOR THE DOWNREGULATION OF IFNγ PRODUCTION

June 22, 2001

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend this application as follows:

IN THE CLAIMS

Cancel claims 1-11 without prejudice, and add claims 12-34, which follow:

Claim 12 A method for treating a subject with a pathological condition involving a gamma interferon mediated effect in progression of said pathological condition, comprising administering an amount of a penicillin sufficient to exert a therapeutic effect on said subject.

Claim 13 The method of claim 12, wherein said pathological condition is an autoimmune disease.

Claim 14 The method of claim 12, wherein said penicillin is penicillin G or penicillin V.

Claim 15 A method for treating a subject with a pathological condition involving a gamma interferon mediated effect in progression of said pathological condition, comprising administering an amount of a hapten modified peptide to said subject sufficient to exert a therapeutic effect on said subject, wherein said hapten modified peptide comprises a backbone of amino acids, wherein a penicillin antibiotic molecule is bound to at least one of said amino acids.

Claim 16 The method of claim 15, wherein said penicillin is penicillin G, penicillin V, or ampicillin.

Claim 17 The method of claim 15, wherein said pathological condition is an autoimmune disease.

Claim 18 A method for determining predisposition of a subject for a hypersensitivity reaction against a penicillin, a part of a penicillin, or a derivative of a penicillin, comprising contacting a sample of peripheral blood monocyte cells from said subject with a hapten modified peptide, said hapten modified peptide comprising a backbone of amino acids and a penicillin antibiotic molecule bound to at least one of said amino acids and measuring the effect of said hapten modified peptides in cells.

Claim 19 The method of claim 18, comprising measuring proliferation of antigen responsive T cells.

Claim 20 The method of claim 18, comprising measuring at least one of gamma interferon expression and interleukin -4 expression.

Claim 21 The method of claim 18, wherein said penicillin is penicillin G, penicillin V, or ampicillin.

Claim 22 The method of claim 18, wherein said backbone of amino acids comprises a lysine residue to which said penicillin antibiotic is bound.

Claim 23 The method of claim 18, wherein said backbone of amino acids comprises a tyrosine anchor, and said penicillin antibiotic is bound to an amino acid at position 3, 5, or 8 from said tyrosine anchor.

Claim 24 A method for desensitization of a subject suffering from a hypersensitivity reaction, comprising administering to said subject a hypersensitivity reaction desensitizing

amount of a hapten modified peptide which contains a backbone of amino acids, wherein a peptide antibiotic molecule is bound to at lest one of said amino acids.

Claim 25 The method of claim 24, wherein said penicillin is penicillin G, penicillin V, or ampicillin.

Claim 26 The method of claim 15, wherein said backbone of amino acids consists of 8-20 amino acids.

Claim 27 The method of claim 25, wherein said backbone of amino acids consists of 10-18 amino acids.

Claim 28 The method of claim 24, wherein said backbone of amino acids consists of 8-20 amino acids.

Claim 29 The method of claim 27, wherein said backbone of amino acids consists of 10-18 amino acids.

Claim 30 The method of claim 15, wherein said backbones of amino acids comprises a lysine residue to which said penicillin antibiotic molecule is bound.

Claim 31 The method of claim 25, wherein said backbone of amino acids comprises a lysine residue to which said penicillin antibiotic molecule is bound.

Claim 32 The method of claim 15, wherein said backbone of amino acids comprises a tyrosine anchor, and said penicillin antibiotic is bound to an amino acids at position 3, 5 or 8 from said tyrosine anchor.

Claim 33 The method of claim 25, wherein said backbone of amino acids comprises a tyrosine anchor, and said penicillin antibiotic is bound to an amino acid at position 3, 5 or 8 from said tyrosine anchor.

Claim 34 The method of claim 29, wherein said backbone of amino acids comprises a tyrosine anchor, and said penicillin antibiotic is bound to an amino acid at position 3, 5, or 8 from said tyrosine anchor.

Claim 35 The method of claim 30, wherein said backbone comprises a tyrosine anchor, and said penicillin antibiotic is bound to an amino acid at position 3, 5, or 8 from said tyrosine anchor.

REMARKS

Entry of the amendment is requested. Claims 12-35 will be pending.

Respectfully submitted,

FULBRIGHT & JAWORSKI L.L.P.

By _____
Norman D. Hanson
Reg. No. 30,946

666 Fifth Avenue
New York, New York 10103
(212) 318-3000

Penicillins as pharmaceuticals for the downregulation of IFNγ production

Specification

The present invention relates to the use of penicillins as therapeutic agents for the treatment of autoimmune diseases or other pathological situations where IFNγ-mediated effects are involved in the progression of the disease and to hapten-modified peptides for use as pharmaceutical compounds and/or diagnostic tools for the determination of a predisposition for hypersensitivity reactions and for the desensitization of patients suffering from hypersensitivity reactions against penicillins or parts or derivatives thereof.

T lymphocytes comprise a central part of the immune system in vertebrates. Their differentiation upon antigenic stimulation plays a critical role in adverse immune responses including autoimmunity and hypersensitivity reactions.

Specific T cell activation is achieved exclusively by interaction of specialized antigen receptors (TCR), clonally distributed on T lymphocytes with presenting molecules, encoded by the major histocompatibility complex (MHC) genes on competent cells, associated to the antigenic stimuli (peptidic antigen). Cytotoxic T cells (Tc) require class I MHC molecules, while helper T cells (Th) need class II MHC molecules. This so-called MHC restricted antigen recognition results from stringent selection processes during T cell maturation in the thymus.

Upon initial exposure to a foreign antigen, T cells can differentiate into type 1 or type 2 phenotype, functionally different. Type 1 cells secrete mainly IFNγ and are involved in cell-mediated adverse reactions. Type 2 cells produce IL4 and drive antibody-mediated immune responses (Ref. 8). In

cases where polarized responses are dominant, the antigen dose appears as one of the critical parameters in determining naive T cell differentiation (Ref. 9). For peptidic antigens, it has been shown that high antigen doses drive IFNγ production and type 1 differentiation of naive T cells, while low doses of the same antigen lead to IL4 secretion and type 2 differentiation.

Using as model the penicillin antibiotics, the inventors examined the modulation of T cell cytokine pattern upon hapten stimulation.

A principal object of the present invention was to provide possibilities to influence the T cell cytokine pattern and to provide pharmaceuticals which can be used to treat patients suffering from diseases which are caused by overreactions or malfunctions of the immune system.

This object is solved according to the invention by the use of penicillins for the treatment of autoimmune diseases or other pathological situations where IFNγ mediated effects are involved in the progression of the disease. In another embodiment of the invention the object is solved by the use of one or more penicillin for the production of a pharmaceutical for the treatment of autoimmune diseases or other pathological situations where IFNγ mediated effects are involved in the progression of the disease.

As already shown for other antigen specific reactivities, also the specific immune response to penicillins is dose dependent, as can be seen from the examples included in this specification. In the context of the present invention, it was possible to show that penicillins can be used successfully to downmodulate IFNγ expression.

For the use according to the invention principally all ß-lactam antibiotics can be used as a penicillin except ampicillin. In preferred embodiments of the invention, Pen V and Pen G are used to block the IFNγ production of ongoing immune responses.

The finding of the invention that penicillins, and preferably Pen V and Pen G, can be used to downmodulate IFNγ production provides a new and promising possibility for therapy of autoimmune diseases or other pathologies where IFNγ mediated effects are involved in the progression of the diseases or at least improvement of the situation of the patients.

A further object ot the invention is the identification of hapten-modified MHC- binding peptides which can be used as molecular antigen inducing MHC-restricted adverse drug-specific immune reactions.

It has long been known that T cells may react to a multitude of chemical reagents, including drugs, often resulting in adverse immune reactions. It was therefore an object ot the present invention to provide compounds that allow to study the interactions of certain drugs and the components of the immune system. It was a further object of such molecules for patients to benefit of their properties. Especially it was desirable to provide diagnositc tools to identify specific T cells that are able to provide possibilities to prevent certain types of allergic reactions.

In this context the invention provides hapten-modified peptides for use for the production of pharmaceutical compounds and/or diagnostic tools, wherein the peptide contains a backbone of amino acids wherein on at least one of these amino acids a penicillin antibiotic is bound.

In preferred embodiments of the invention, the penicillin antibiotic is Pen G, Pen V or ampicillin (amp).

It was discovered (Ref. 6, 7) that hapten-modified peptides which are to be used according to the invention are especially suitable when their amino acid backbone consists of 8 to 20 amino acids, preferably 10 to 15 amino acids. In a further preferred embodiment the amino acid backbone contains at least one lysin onto which the antibiotic is bound. In still a further

preferred embodiment the hapten-modified peptide contains a tyrosine on the amino acid backbone. The tyrosine is preferably present, since it serves as anchor to the MHC molecules. Once the hapten-modified peptide is anchored to the MHC molecule, it has the right conformation to contact and eventually bind specific TCR.

In the further preferred embodiment the lysin onto which the antibiotic is bound is located in position 3, 5 or 8 calculated from the tyrosine anchor on the amino acid backbone.

The hapten-modified peptides according to the invention are useful as pharmaceutical compounds and/or diagnostic tools. One diagnostic application for the hapten modified peptides is the screening of patients for a predisposition for hypersensitivity reactions against penicillins in general. In a preferred embodiment of the invention PBMCs (peripheral blood mononuclear cells) are isolated from patients with suspected predisposition of allergic reactions. PBMCs are easily purified from the blood of patients. They are stimulated in vitro by addition of the hapten-modified peptides according to the invention and the proliferation of antigen responsive T cells is measured.

In view of the fact that such responsive T cells bind via their TCR to the hapten-modified peptides, such T cells can be detected by an immunoassay using the hapten-modified peptides as capture reagent. Immunoassay protocols for conducting such assays are known in the art and can easily be adjusted to the present object.

The invention, however, is not to be restricted to such immunoassays but every other possibility to measure proliferation of antigen responsive T cells is also encompassed by the present invention.

Since such proliferating T cells produce IFNγ and IL4 a further preferred possibility to measure proliferation lies in the monitoring of production of these substances.

Since the hapten-modified peptides according to the invention very potently stimulate T cell proliferation a further use of these substances lies in their application as pharmaceuticals for the desensitization of patient suffering from hypersensitivity reactions against penicillins or parts or derivatives thereof. This use for desensitization is a further object of the invention.

It is easily feasible to combine the peptides according to the invention with penicillin haptens, against which the allergic reaction is directed. Therefore, for patients that show hypersensitivity against for example Pen G, Pen G is used as hapten.

The hapten-modified peptides according to the invention can easily be produced in pure form. This enables a safe desensitization without the risk of further adverse reactions to the antibiotic molecule which, once covalently bound to the peptide, looses its chemical reactivity. The production of the hapten-modified peptides also is not expensive and the overall desensitization might become much cheaper as it is at present.

It is also preferred to use parts or derivatives of the penicillins for the desensitization treatment. Such parts of the molecule might be useful for the hyposensitization, but might not be as immunogenic as the complete drug molecule itself. Only parts of the penicillin molecules can therefore provide a possibility of safer and less radical treatments.

The following examples together with the figures are intended to further illustrate the invention.

Example

1. Antigens

Pen G, Pen V and Amp were obtained from Sigma, St. Louis, MO. PHA was from Murex Diagnostics Ltd., Dartford, UK. The tetanous toxoid peptide 616-630 (VRDIIDDFTNESSQK) was synthesized by continuous-flow solid-phase peptide synthesis. For all antigens, a 10-fold concentrated stock solution was freshly prepared in PBS and added to the wells at the time of experiment.

2. Culture media

The complete culture medium used in this study was RPMI 1640 supplemented with 2 mM L-glutamine, 5 x 10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate and 1x mixture of non-essential amino acids (Gibco BRL, Paisley, Scotland). The medium was prepared without penicillins or any other antibiotics. For growing T cell clones the complete RPMI medium was used with addition of 5% pooled human AB serum (HS; Swiss Red Cross, Bern Switzerland) and 250 U/ml of recombinant human IL2 (Proleukin, EuroCetus, Ratingen, Germany). EBV-transformed B-lymphoblastoid cell lines were grown in complete RPMI supplemented with 10% heat-inactivated fetal calf serum.

3. Primary culture

Donors BAM, CAS and ES had a positive history of penicillin allergy and their clinical data are summarized in Table I. Primary cultures were prepared from freshly isolated PBMC in two parallel 96 well plates. Cells at a concentration of 3 x 10^5/w were stimulated with 3-fold dilutions of Pen G from 3 mg/ml to 0.1 μg/ml. After 5 days incubation at 37°C in 5% CO_2, the proliferative response was determined, in one of the plates, as the ^{3}H-thymidine incorporation after 16 h incubation with 1 mCi = 37 kBq ^{3}H-thymidine (Dupont, Boston, MA). Cells were harvested on GF/A-filters (Dunn Labortechnik GmbH, Asbach, Germany) and the incorporation was

measured in an automatic β-counter (Inotech, Asbach, Germany). The second plate was further expanded in IL2-containing medium for 4 days. Cells were then pooled for any given Pen G concentration, washed and rested in the absence of IL2. After another 4 days, cells were harvested and analysed for TCR expression by cell surface staining and IL4/IFNγ production by intracellular staining.

4. T cell clones

T cell clones were generated from Pen G specific T cell lines. Freshly isolated PBMC were stimulated with 1, 0.3, 0.1 mg/ml of Pen G at a density of 2 x 10^5 cells/well in a final volume of 200 µl of complete RPMI containing 5 % HS in 96-well flat bottom culture plates. After 5 days at 37°C and 5 % CO_2, 25 U/ml of IL2 was added to the wells. After another 4 days the cultures were pooled, rested for 3 days in the absence of IL2 and subjected to a second round of restimulation using irradiated autologous PBMC as APC and the same dose of Pen G given during the first restimulation. After 3 days the different cultures were again expanded in IL2 containing medium and then cloned by limiting dilution. T cell blasts were seeded at 0.3 cells/well in Terasaki plates (Nunc, Roskilde, Denmark) in the presence of 1 µg/ml PHA and 10^4 irradiated allogeneic PBMC in complete RPMI medium containing 5 % HS and 250 U/ml IL-2. The T cell clones obtained were maintained in culture by periodic stimulation in the presence of irradiated allogeneic PBMC, PHA and IL2.

5. **EBV-transformed B cells**

Autologous EBV-transformed B-lymphoblastoid cell lines (EBV-B cells) were prepared by culturing fresh isolated PBMC in complete RPMI containing 10 % FCS, 30 % supernatant of the EBV-producing marmoset cell line B95-8 (American Type Culture Collection, ATCC, Rockville, MD) and 600 ng/ml cyclosporin A (Sandoz, Basel, Switzerland). After overnight incubation, cells were washed and cultured in complete culture medium.

6. **Flow cytometric analysis of intracellular IFNγ and IL4 production**

Intracellular staining for cytokines was performed following the protocol described by Murphy et al. (12), with few modifications. Briefly, cells were resuspended at 10^6/ml in 96 well plates in a final volume of 200 µl/w of culture medium and stimulated with PMA at 50 ng/ml plus ionomycin at 500 ng/ml for 5 h at 37°C. Brefeldin A at 10 µg/ml was then added to the wells and incubation was prolonged for another 4 h. Cells were then washed and resuspended in 100 µl/w of PBS before adding an equal volume of 4 % formaldehyde fixative. After fixing for 20 min at room temperature, cells were either stored at 4°C over night or stained immediately for intracellular cytokines. For intracellular staining, all reagents and washes contained 1 % BSA and 0.5 % saponin, and all incubations were at room temperature. Cells were first washed and permeabilized for 30 min. After washing, blocking mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Gove, PA) at a concentration of 300 µg/ml were given to the well for 10 min. Then, PE-conjugated anti human IL4 antibody (Clone 8D4-8, Pharmingen, San Diego, CA) plus FITC-conjugated anti human IFNγ antibody (Clone 4S.B3, Pharmingen) or isotype matched controls (PE- and FITC-conjugated mouse IgG1, Pharmingen) at a final concentration of 5 mg/ml, were directly added to the wells. After 20 min, cells were washed twice in PBS/BSA/saponin and then with PBS/BSA to allow membrane closure. Samples were resuspended in PBS/BSA and analyzed on a FACScan flow cytometer (Becton Dickinson & Co.). Thresholds were set on control stainings, included for every sample; 50,000 cells/sample were acquired. Results were analyzed using Cellquest software. T cells were detected by standard surface staining using the PE-conjugated anti human TCRαβ antibody (Clone BMA03, Immunotech, Marseille, France).

7. **Cytokine measurement**

For cytokine measurement, 10^5 T cells and 5 x 10^4 autologous, irradiated EBV-B cells were mixed in 200 µl of complete RPMI-FCS medium containing appropriate Ag concentration. After overnight incubation, supernatants were

collected and tested for the concentration of IL2, IFNγ and IL4. IL2 was quantified on a IL2-dependent CTLL line. IFNγ was measured by a sandwich ELISA as described (12). IL-4 was measured using the Duo-Set ELISA kit provided by Genzyme Diagnostics, Cambridge, MA. Eventually, cells were kept in culture for another 24 h and cellular proliferation was measured by thymidine uptake as described above.

8. Preferential expansion of PBMC-derived IL4-producing cells upon stimulation with different doses of Pen G

Patients BAM, CAS and ES participating to this study developed allergic reactions after treatment with β-lactam antibiotics and their clinical data are summarized in Table I. At the time of diagnosis they all presented different symptoms after treatment with β-lactams as well as positive LTT in vitro (Table I; ref. 2, 4 and data not shown). The Pen G specificity of their allergic reactions was assessed by RAST and skin tests which gave clear positive results for donors BAM and ES, but were negative for donor CAS. For all the three donors an immediate type of hypersensitivity was diagnosed, associated to delayed type reactions for donor BAM. Primary in vitro cultures were prepared stimulating purified PBMC with different doses of Pen G. The hapten was given in three fold dilutions starting from 3 mg/ml down to 0.1 μg/ml concentration, in two parallel cultures. After 5 days of incubation, the proliferative response was measured in one of the cultures by thymidine incorporation. As shown in Fig. 1, PBMC proliferated in response to Pen G in the range from 1 to 0.01 mg/ml, depending on the donor. The intensity of the response was dose-dependent, with maximal proliferation at 0.3 mg/ml of Pen G.

The second culture was further expanded in IL2-containing medium for 4 days. Cells were then washed and rested in the absence of IL2. After another 4 days, cells were harvested, restimulated with PMA and ionomycin, and analysed for intracellular IFNγ and IL4 expression by flow cytometry. TCRαβ$^+$ cells constituted up to 90 % of the cell populations and

they were homogeneously represented in the different cell lines (Fig. 1). As shown in the right panels of Fig. 1, upon in vitro stimulation with Pen G, mainly $IL4^+$ and $IL4^+IFN\gamma^+$ cells are expanded, while the growth of IFNγ-producing cells was rather unaffected. Comparing left and right panels of Fig. 1, the expansion of IL4-producing cells directly correlated with the intensity of the proliferative response. This indicating that, in our system, Pen G preferentially induced the recruitment of IL4-producing cells from peripheral blood lymphocytes and the intensity of their expansion depended on the dose of hapten used to induce the primary response in vitro. Interestingly, the preferential expansion of Pen G-specific, IL4-producing cells was associated to immediated type of hypersensitivity reactions in all the patients analyzed (Table I).

9. Effect of "hapten doses" on T cell clones

In order to evaluate the role of Pen G doses in inducing functional differentiation of established T lymphocytes, a panel of independent Pen G-specific T cell clones was prepared. PBMC from donors BAM and ES were subjected to two rounds of stimulation, in vitro, with Pen G at concentrations of 1, 0.3 and 0.1 mg/ml in three independent T cell lines. After expansion in IL2-containing medium, each line was independently cloned by limiting dilution. A total of 18 Pen G-specific T cell clones were obtained from donor BAM and 24 from donor ES. The hapten-specific T cell phenotype was induced stimulating each T cell clone with 3-fold dilution of Pen G, from 3 mg/ml to 0.03 mg/ml concentration, in the presence of irradiated autologous EBV-B cells. After 24 h incubation, supernatants were harvested and processed for cytokine measurement, while cells were kept in culture for another 24 h and T cell proliferation was measured by thymidine uptake. We then examined IL4 and IFNγ secretion induced in the presence of the highest and lowest Pen G dose able to trigger cellular proliferation, considering SI values ≥ 2.0 as positive response. Results are reported in Table II.

Clones from donor BAM showed strong proliferation in response to the highest dose of hapten (3 mg/ml), with SI values up to 122.7. For these clones the lowest dose of Pen G capable of triggering cellular proliferation was 0.03 mg/ml and, even at this low concentration, SI values of proliferation wer up to 86.5, indicating the high sensitivity of these reactivities. Phenotypically, BAM clones expressed a strong preference for type 0 or type 1 responses, and half of the clones were able to secrete both IL4 and IFNγ at high and low hapten doses. Interestingly, lower Pen G concentration induced higher level of IFNγ secretion in 14 of the 18 clones analyzed. This dose-dependent cytokine pattern resulted in a hapten-specific modulation of the T cell phenotype, and remarkably, 5 of the clones analyzed were able to shift from type 0 to type 1 responses.

Clones from donor ES resulted less sensitive to the hapten, being unable to proliferate to Pen G at concentrations < 0.3 mg/ml. For these the lowest Pen G doses capable of triggering cellular proliferation were ≥ 0.3 mg/ml with SI never exceeding 8.6. Clones ES mounted type 2 responses but could secrete IL4 only at the highest dose of Pen G tested (3 mg/ml).

Altogether, the hapten-specific T cell phenotypes were independent of the dose of Pen G used during in vitro induction of PBMC but depended on the donor analyzed. Thus, from donor BAM, who suffered of delayed as well as immediate type of hypersensitivity reactions, it was possible to isolate, after two cycles of restimulation with the specific hapten, IFNγ-producing T cell clones. In contrast, from donor ES, characterized by immediated type of hypersensitivity, mainly IL4-producing T cell clones could be isolated. However, the concentration of Pen G was indeed capable of modulating the T cell phenotypes. In fact, very high doses of Pen G were required to induce IL4 production by ES clones, while low doses of hapten induced higher level of IFNγ secretion, resulting in a shift of T cell phenotypes.

10. Pen G can modulate the T cell phenotype of fully differentiated hapten-specific T cell clones

To rule out if the antigen-driven phenotype modulation was a peculiar characteristic of the hapten under investigation, we further examined the effect of "antigen doses" on two other $CD4^+$ T cell clones.

Clone BAM25 was isolated 2 years ago in our laboratory and has been already described as Pen G-specific (7). clone 1ES4 derived from stimulation of peripheral blood cells of donor ES with the tetanus toxoid peptide sequence 616-630. The two clones were stimulated in vitro with different concentrations of either the specific Ag or the T cell mitogen PHA and cytokines released in the culture supernatant were measured after 24 h incubation.

As shown in Fig. 2, the cytokine patterns of clone BAM25 depended on the dose of Pen G used for stimulation. This clone was raised with a dose of Pen G of 1 mg/ml (7) and behaved, in this condition, as a Th2 clone, secreting IL4 but not IFNγ. However, the same clone simultaneously secreted IL4 and IFNγ in the presence of lower doses (< 0.3 mg/ml) of Pen G (Fig. 2A). In contrast, PHA stimulation induced a dose-dependent production of both IL4 and IFNγ (Fig. 2B), indicating the importance of the hapten-specific stimulation in defining Th0 or Th2 profiles. On the other hand, clone 1ES4 secreted IFNγ in response to both the specific antigen as well as the mitogen in a dose-dependent way (Fig. 2C-D).

Thus, the modulation of the T cell phenotype observed seems to be a peculiar characteristic of our hapten system. It appears feasible to drive the cytokine profile of long-term established Pen G-reactive T cell clones, altering the dose of hapten used for in vitro stimulation.

11. Antigen-independent IFNγ downmodulation by Pen G and V

The possibility to modulate the phenotype even of fully differentiated T cell clones could certainly introduce new strategies to manipulate immune responses. However, the data presented above apply exclusively to Pen G specificities. In order to find a system of more general application, the inventors also investigated the possibility to use other penicillin derivatives in addition to Pen G to modulate different antigen-specific immune reactions.

In a first set of experiments we studied the effect of the Pen V and Amp derivatives on the T cell phenotype induced by Pen G on two hapten-specific $CD4^+$ cell clones. Upon stimulation with 0.25 mg/ml of Pen G, clone BAM25 secreted IL2, IL4 and IFNγ (Fig. 3A-C), while clone ES5.13 produced IL2 and IL4 (Fig. 4A-B). None of them responded to neither Pen V nor Amp (Fig. 3A-C and Fig. 4A-B, respectively). In the assay, graded concentrations of Pen V and Amp were used together with 0.25 mg/ml of Pen G for stimulating T cell clones and the cytokines secreted in the culture supernatants were measured after 24 h incubation.

Results obtained from clone BAM25 are shown in Fig. 4D-F. Addition of Amp did not affect the Pen G-induced cytokine pattern, except for a slight reduction of IFNγ secretion at high concentration (Fig. 3D-F). In contrast, Pen V reduced the amount of IL2 of about 50 % (Fig. 3D) and completely abrogated IFNγ production at concentrations $\geq$ 0.1 mg/ml, without affecting IL4 secretion (Fig. 3E-F). The same type of assay, performed on clone ES5.13, revealed no interference of either Pen V or Amp on Pen G-induced IL2 secretion (Fig. 4C) and only a marginal reduction of IL4 production with high Pen V concentration (Fig. 4D).

Considering the structural similarities of the different penicillin molecules one might expect that the strong effect on IFNγ downregulation observed with Pen V could be due to altered interactions with the antigen-binding

sites of penicillin-specific TCR. In order to determine if this was the case, we performed the same assay described above, with the tetanus toxoid-specific T cell clone 1ES4. Upon stimulation with either the peptide sequence TT 616-630 at 1μg/ml and PHA at 0.3 μg/ml, this clone has a classical Th1 phenotype (Fig. 2). Surprisingly, even for these reactivities, the addition of Pen V at $\geq$ 0.1 mg/ml completely abrogated IFNγ secretion in response to either the specific antigen (TT peptide 616-630, Fig. 5B) and the mitogen (PHA, Fig. 5D), while IL2 production was unaffected (Fig. 5A,C). Again, addition of Amp had no effect (Fig. 5).

Thus, a complete inhibition of IFNγ secretion could be induced by addition of Pen V on two T cell clones of totally different specificities, i.e. Pen G (clone BAM25) or tetanus toxoid (clone 1ES4). Therefore, the effect observed is more likely independent of interaction with the TCR, but rather due to a direct interference of Pen V with IFNγ secretion pathways.

Downmodulation of the IFNγ production by PHA-stimulated T cells could be achieved also upon addition of Pen G, as shown in Fig.6. Similarly to what previously shown for Pen V, the IL-2 secretion was unaffected (Fig. 6, A and B). Amp, tested in the same experiment, showed again no effect on both IFNγ- and IL-2 secretion.

12. Implications

It has long been known that antigen dose can influence whether cell mediated or humoral responses are elicited and this largely depends on the development of $CD4^+$ T lymphocytes producing distinct sets of cytokines (8). In the mouse system, very high doses of antigenic peptides direct the differentiation of naive T cells into IL4-producing cells, while low levels of antigen promote the expansion of IFNγ-producing cells (9, 10). These findings suggested that antigen dose can alter the cytokine synthesis in naive $CD4^+$ T cells and even the induction and progression of drug-specific

adverse immune responses could be determined at the primary encounter with the hapten.

In the present examples the inventors focussed on the human allergic response to β-lactam antibiotics investigating the influence of hapten doses first, on the recruitment of drug-specific cells from PBMC of allergic donors, secondly, on the induction of T cell phenotype of established T cell clones.

All the donors participating in this study suffered from immediated type of hypersensitivity to β-lactams, and in particular to Pen G. Upon in vitro stimulation with different doses of Pen G we found a preferential recruitment of IL4-producing peripheral blood-derived T cells. This expansion was maximal for high-middle doses of hapten (around 1-0.1 mg/ml concentration) and directly correlated with the proliferative response of the cultures. This suggests that the drug-specific cells contributing to the adverse reactions are mainly of type 2 phenotype, as expected from the clinical characteristics of all the donors. Remarkably, this effect was particularly evident for donor CAS who developed an anaphylactic shock after treatment with β-lactams.

In those experiments the inventors were able to analyze only the contribution of memory T cells to penicillin-specific allergic responses. To rule out how these drug-specific reactions are induced at the very early encounter with the hapten and how hapten concentrations might influence T cell differentiation, it could be certainly interesting to investigate the effect of Pen G on human cord blood-derived naive T cells.

The role of Pen G doses in inducing functional differentiation of established T lymphocytes was analyzed on a panel of 42 independent T cell clones isolated from Pen G-specific after two cycles of restimulation with the hapten. It was found that the concentration of Pen G during in vitro induction does not affect the type of clones isolated, this depending only

on the donor studies. However, once T cell clones have been isolated and expanded, the dose of Pen G used for stimulation was, indeed, a critical factor determining their functional, hapten-specific phenotype. In fact, only high doses of Pen G (3 mg/ml) induced IL4 secretion and the type 2 clones isolated behaved as "inert" cells at low hapten concentration. On the other hand, low Pen G concentrations induced higher level of IFNγ secretion; as a consequence, 35% of clones isolated from donor BAM were able to shift their phenotype from type 0 to type 1 (Table II). Remarkably, the phenotype shift was very peculiar for penicillin-specific responses, since it was not observed with a tetanus toxoid-specific $CD4^+$ clone (i.e. clone 1ES4) and even not by PHA stimulation (Fig. 2). Moreover, the phenomena observed depended mainly on the modulation of IFNγ secretion as shown in detail for clone BAM25 which could secrete IFNγ only at Pen G concentration ≤ 0.3 mg/ml (Fig. 2).

For a correct interpretation of these data, one should consider how hapten-derived epitopes are formed and how the strength of their interaction with specific TCR is determined, in comparison to nominal peptidic Ag (13, 14). On a given APC, the density of epitopes formed by hapten-modification will depend, at least, on two factors: the number of modifiable sites and the concentration of reactive hapten molecules. At saturation, the density of epitopes will not increase with the concentration of the hapten. On the other hand, multiple modification might interfere with the strength of TCR ligation. We already found that a peptide sequence carrying multiple penicilloyl groups was a poor stimulator compared to a designer penicilloyl-peptide carrying only the preferential modified lysin residue, for the same clone (15). Taken together, these considerations suggest that for drug-specific immune responses there is no direct correlation between concentration of hapten molecules, epitope density and strength of TCR engagement.

Several recent studies focus on the identification of factors and signals capable of reversing Th responses (16, 17). In the inventor's system, the phenotype-modulation observed for Pen G-specific immune responses is, most probably, TCR-mediated.

In the last set of experiments presented, it was possible also to identify at least two penicillin derivatives capable of inducing a modulation of the T cell phenotype, with a TCR-independent mechanism. In fact, a dose-dependent inhibition of IFNγ production was observed when Pen V was added to manipulate either a Pen G-specific immune response (clone BAM25 in Fig. 3) as well as a tetanus toxoid-specific response or even a mitogen-induced phenotype (clone 1ES4 in Fig. 5). Similarly, also Pen G could downmodulate IFNγ production of mitogen-activated T cells (Fig. 6). In all these systems a downregulation of IFNγ secretion was observed, with no effect on IL4 production. The broad range of specificities which could be manipulated speaks in favour of TCR-independent mechanisms for Pen V-mediated IFNγ downmodulation. A similar effect has been very recently described for other compounds, such as pirydinil imidazole drugs. One of these compounds, SB203580, selectively induces IFNγ downmodulation in mouse Th1 cells through specific inhibition of p38 MAP kinases (18). It is tempting to speculate that similar targets could be modified also by Pen V.

Taken together, the above data indicate that penicillins can modulate the T cell phenotype of fully differentiated T cell clones in vitro, with TCR-dependent and TCR-independent mechanisms, thus representing potentially new tools for immune intervention. In particular, the possibility to selectively downmodulate IFNγ production via TCR-independent pathways offers a broad application for therapeutic intervention in autoimmune diseases and other pathological situations where a predominant Th1 profile is found.

Figure 1

Pen G-specific response of PBMC from donors allergic to β-lactams: effect of hapten doses on the recruitment of IL4-producing cells. Purified PBMC from donors BAM, CAS and ES were cultured, in vitro, with different doses of Pen G in two parallel cultures. Proliferative responses measured on the first culture are shown in the left panels; data are expressed in cpm x 10^3 and given as mean of triplicates, SD values are indicated. Cells in the second culture were further expanded, then rested in the absence of IL2 and finally harvested, restimulated with PMA and ionomycin, and analysed for IFNγ and IL4 productions by flow cytometry. Data are shown in the right panels and represented as percentage of IL4 (black bars), IFNγ (white bars) and IL4/IFNγ (grey bars) producing cells. For all the cultures the frequency of TCR$\alpha\beta^+$ cells, reported on the right, was determined by surface staining.

Figure 2

Pen G can modulate the phenotype of an established $CD4^+$ hapten-specific T cell clone. Clones BAM25 (A,B) and 1ES4 (C,D) were analysed for cytokine secretion in response to different concentrations of the specific antigen, Pen G (A) or TT616-630 peptide (C) respectively, and PHA (B,D). IFNγ and IL4 productions are shown in the left and right panels respectively. Data are expressed as pg/ml of secreted cytokines and given as mean of triplicates, SD values are indicated.

Figure 3

Effect of Pen V and Amp on the Pen G-specific phenotype of clone BAM25. IL2, IL4 and IFNγ productions in response to different concentration of Pen G (square), Pen V (circle) and Amp (triangle) are represented in A, B and C, respectively. In the experiment shown in the right panels, 10^5 T cells/w and 5 x 10^4 EBV-B cells/w were stimulated with 0.25 mg/ml of Pen G plus different amount of Pen V or Amp. IL2, IL4 and IFNγ productions, measured after 24 h incubation, are shown in D, E and F, respectively. Data are given as mean of triplicates and represented as % of cytokines secreted in the

presence of Pen G only. 100% values were: IL2 80.5 SI of CTLL cells, IL4 5788 pg/ml, IFNγ 6637 pg/ml.

Figure 4

Effect of Pen V and Amp on the Pen G-induced phenotype of clone ES5.13. IL2 and IL4 productions in response to different concentrations of Pen G (square), Pen V (circle) and Amp (triangle) are shown in A and B, respectively. The assay, shown in the right panels, was performed as described in Fig. 3. IL2 and IL4 productions, shown in C and D respectively are given as mean of triplicates and represented as % of cytokines secreted in the presence of Pen G only. 100% values were: IL2 40.3 SI, IL4 5414 pg/ml.

Figure 5

TCR-independent IFNγ downmodulation by Pen V. Clone 1ES4 was stimulated with the tetanus toxoid peptide sequence 616-630 at 1μg/ml (A,B) or PHA at 0.3 μg/ml (C,D) plus different doses of Pen V and Amp as described in Figure 3. IL2 and IL4 secretion after 24 h incubation are represented in A, C and B, D, respectively. 100 % values for TT 616-630 were: IL2 137.4 SI of CTLL cells, IFNγ 7497 pg/ml; for PHA: IL2 116 SI, IFNγ 7865 pg/ml.

Figure 6

Effects of penicillins on IFNγ production by fresh peripheral blood cells (PBMC).

Ficoll-purified PBMC ($1x10^5$) of a healthy donor were incubated with 1μg/ml phytohemeagglutinin (PHA) in the absence or presence of penicillin G (Pen G), penicillin V (Pen V) or Ampicillin (Amp) at the indicated concentrations. After 24 h at 37°C and 5% CO_2, supernatants were collected and IFNγ (A) or IL-2 (B) determined by ELISA. Maximal concentrations in the absence of antibiotics were 3.000 pg/ml for IFNγ and 600 pg/ml for IL-2. As shown, none of the antibiotics significantly affects IL-2 secretion while IFNγ

production is blocked almost completely by high concentrations of Pen G and Pen V, but not by Amp.

Table I Characteristics of the allergic individuals studied

Donor	Symptoms	IgE[a]	Skin Tests		Type of hyper-sensitivity
			Immediate[b]	Late[c]	
BAM	Urticaria Exanthema	+	-	+	I[e] and D[f]
CAS	Anaphylaxis	-	-	-	I
ES	Eodema	+	+	n.t.[d]	I

[a] Specific serum IgE as determined by RAST.

[b] Prick and i.d. tests for Pen G using either benzylpenicilloyl-polylysin or minor-determinant-mixture (MDM:benzylpenicilloyl/Pen G mixture).

[c] Patch test read after 48 h and 72 h.

[d] n.t. = not tested.

[e] I = immediated type hypersensitivity reaction.

[f] D = delayed type hypersensitivity reaction.

Table II Phenotypes of penicillin-specific T cell clones[a]

Clone[c]		PR (SI)[b]		Cytokines (ng/ml)				Type	
				IL4		IFNγ			
		max[d]	min[e]	max	min	max	min	max	min
BAM G1	B2	63.1	25.7	1.1	<[h]	7.6	8.8	T0	T1
	C12	87.7	56.2	4.1	2.2	6.5	8.6	T0	T0
	B10	46.5	33.0	6.6	4.6	6.4	7.9	T0	T0
	D6	41.2	4.8	1.7	<	5.2	5.9	T0	T1
	B12	81.8	6.7	5.5	4.1	6.6	8.2	T0	T0
	D7	68.3	48.6	1.3	0.5	8.4	8.6	T0	T0
	A10	101.6	18.8	4.2	<	7.6	<	T0	T0
	A6	95.4	81.2	5.0	4.2	8.6	10	T0	T0
	D10	122.7	86.5	7.5	6.9	7.2	8.7	T0	T0
BAM G0.3	D9	13.0	9.1	<	<	0.5	8.6	T1	T1
	D12	25.9	5.7	0.2	0.2	7	8.2	T0	T0
	B8	17.3	14.0	<	<	<	8.6	T0	T1
	C3	70.5	37.0	0.8	0.2	7.6	9.7	T0	T0
	A1	37.3	5.5	0.7	<	4.0	1.7	T0	T1
BAM G0.1	G11	75.4	9.5	<	<	7.1	7.9	T1	T1
	G4	56.4	12.2	7	<	3.4	5.4	T0	T1
	F3	77.1	7.1	10.8	<	<	<	T2	-
	F2	108.0	7.3	4.9	0.5	7.9	7.4	T0	T0
ES G1	A9	76.8	6.3	23.2	<	<	<	T2	-
	C6	16.4	7.6	<	<	<	<	T	-
	A8	19.0	3.0	1.0	<	<	<	T2	-
G0.3	F9	37.6	6.3	<	<	<	<	-	-
	F8	99.6	4.0	2.7	<	<	<	T2	-
	E12	90.7	5.3	1.7	<	<	<	T2	-
	H6	27.2	6.3	<	<	<	<	-	-
	H1	33.1	4.1	<	<	<	<	-	-
	E6	26.2	4.4	<	<	<	<	-	-
	E8	52.6	8.6	<	<	<	<	-	-
	H2	18.5	2.7	<	<	<	<	-	-
	H3	36.7	5.2	<	<	<	<	-	-
	F3	43.5	5.9	1.9	<	<	<	T2	-
	H10	72.4	3.2	<	<	<	<	-	-
G0.1	E6	24.3	2.0	<	<	<	<	-	-
	H3	91.6	6.7	2.7	<	<	<	T2	-
	H11	101.9	2.8	27.9	<	<	<	T2	-
	G2	105.1	6.3	<	<	<	<	-	-
	G4	141.0	5.0	5.3	<	<	<	T2	-
	H10	162.1	8.1	4.6	<	<	<	T2	-
	H8	32.0	3.2	1.1	<	<	<	T2	-
	E7	195.8	3.7	3.1	<	<	<	T2	-
	H6	50.6	4.1	2.7	<	<	<	T2	-
	H7	70.2	2.2	0.9	<	<	<	T2	-

[a] The in vitro test for cytokine production was set up in 96-well plates with 10^5 T cells, 5 x 10^4 irradiated autologous EBV-B and 3 fold dilutions of Pen

G from 3 mg/ml to 0.03 mg/ml. After 24 h supernatants were harvested for cytokine measurement, while cells were kept in culture for another 24 h and T cell proliferation was measured by thymidine inrorporation. The values reported refer to cytokine production in the presence of the highest (max) and lowest (min) hapten doses able to trigger cellular proliferation.

[b] PR: proliferative response; SI: stimulation index.

[c] The name of the clones indicate: the donor of origin, the dose of Pen G used for in vitro priming and the personal code.

[d] max: highest Pen G concentration tested; 3 mg/ml for all clones.

[e] min: lowest Pen G dose able to trigger cellular proliferation; 0.03 mg/ml for BAM clones; ≥ 0.3 for clones ES.

[f] <: not detectable.

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Claims

1. Use of at least one penicillin for the production of pharmaceutical for therapeutic use in autoimmune diseases or other pathological situations where IFNγ-mediated effects are involved in the progression of the disease.

2. Use according to claim 1, wherein the penicillin is PenG or PenV.

3. Use of a hapten-modified peptide for the production of a pharmaceutical for therapeutic use in autoimmune diseases or other pathological situations where IFNγ-mediated effects are involved in the progression of the disease
characterized in that it contains a backbone of amino acids wherein to at least one of these amino acids a penicillin antibiotic is bound.

4. Use of a hapten-modified peptide for the diagnostic determination of a predisposition for hypersensitivity reactions against penicillins or parts or derivatives thereof,
characterized in that it contains a backbone of amino acids wherein on at least one of these amino acids a penicillin antibiotic is bound.

5. Use according to claim 4, wherein PBMC cells are isolated from patients for whom a predisposition for hypersensitivity reactions is to be determined, the cells are stimulated in vitro by adition of the hapten-modified peptide and proliferation of antigen responsive T cells is measured.

6. Use according to claims 4 or 5, wherein IFNγ and/or IL4 expression are measured.

7. Use of a hapten-modified peptide for the desensitization of patients suffering from hypersensitivity reactions against penicillins or parts of derivatives thereof
characterized in that it contains a backbone of amino acids wherein on at least one of these amino acids a penicillin antibiotic is bound.

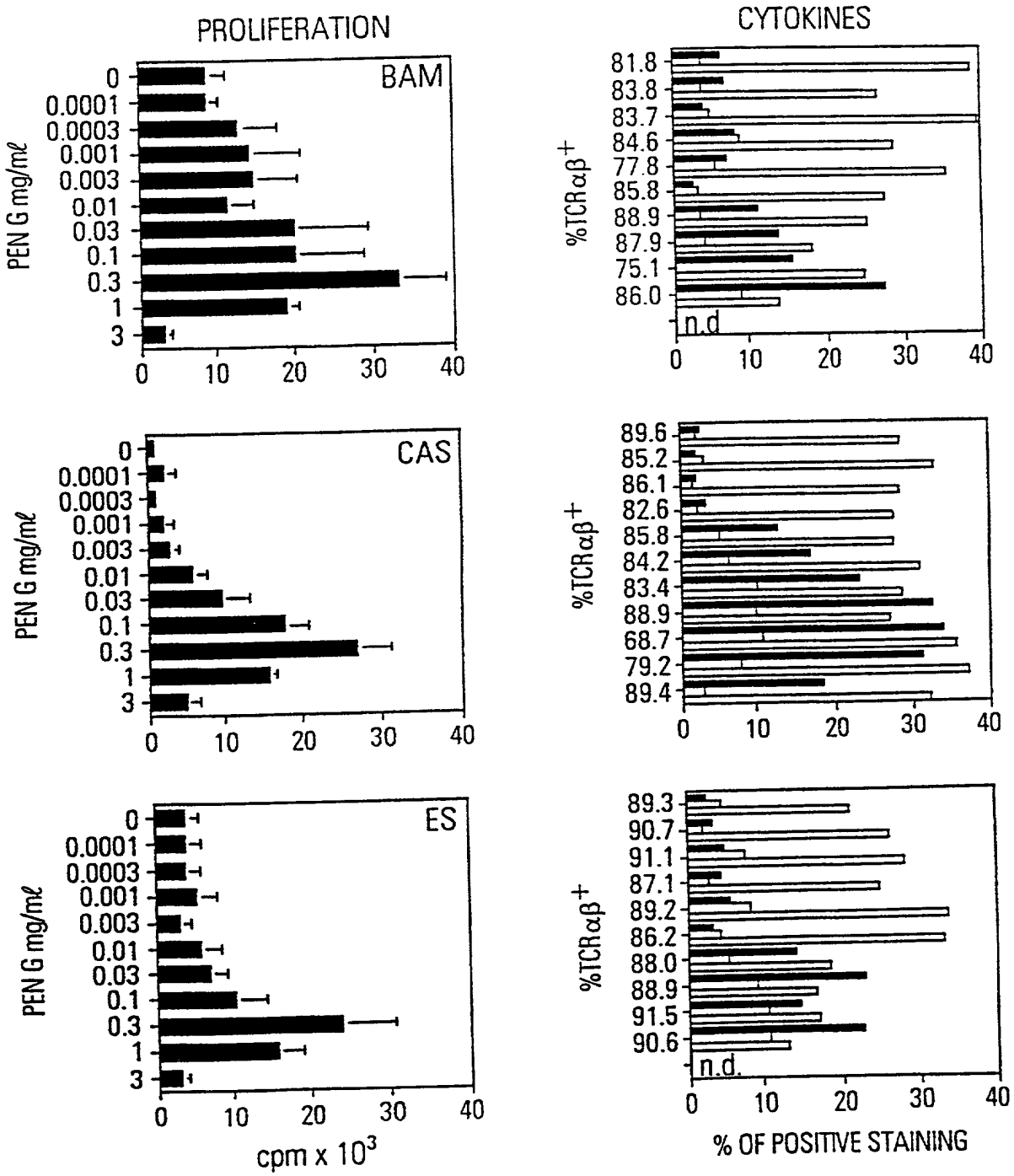
8. Use according to anyone of claims 3 to 7, wherein the penicillin antibiotic is penicillin G, penicillin V or ampicillin.

9. Use according to anyone of claims 3 to 8, wherein the amino acid backbone consists of 8 to 20 amino acids, preferably 10 to 18 amino acids.

10. Use according to anyone of claims 3 to 9, wherein the amino acid backbone contains at least one lysine onto which the antibiotic is bound.

11. Use according to anyone of claims 3 to 10, wherein the antibiotic is bound to amino acids at position 3, 5 or 8 starting from a tyrosine anchor.

FIG.1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0.1
0.3
1
3
%TCRαβ+
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83.8
83.7
84.6
77.8
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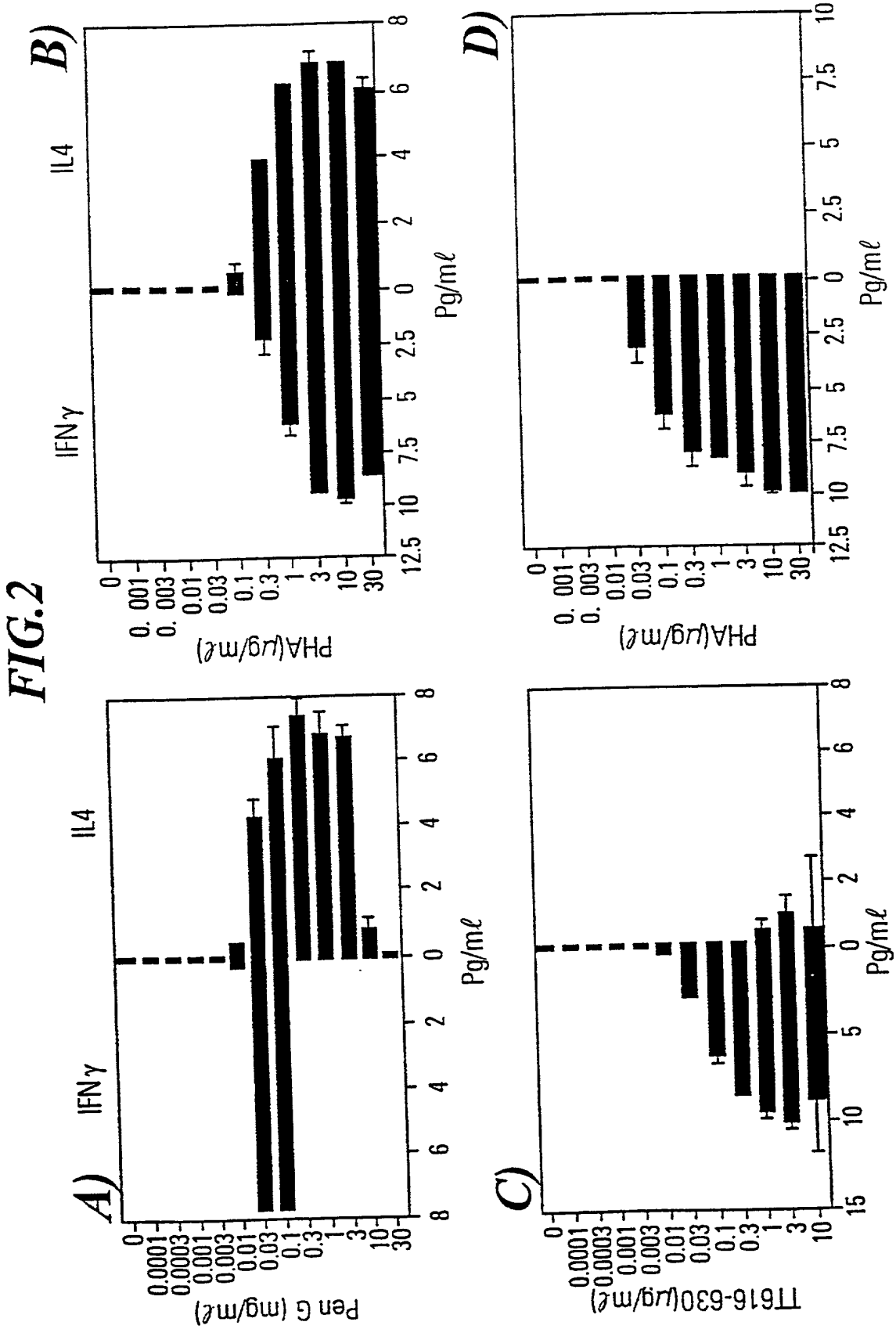
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IFNγ
IL4
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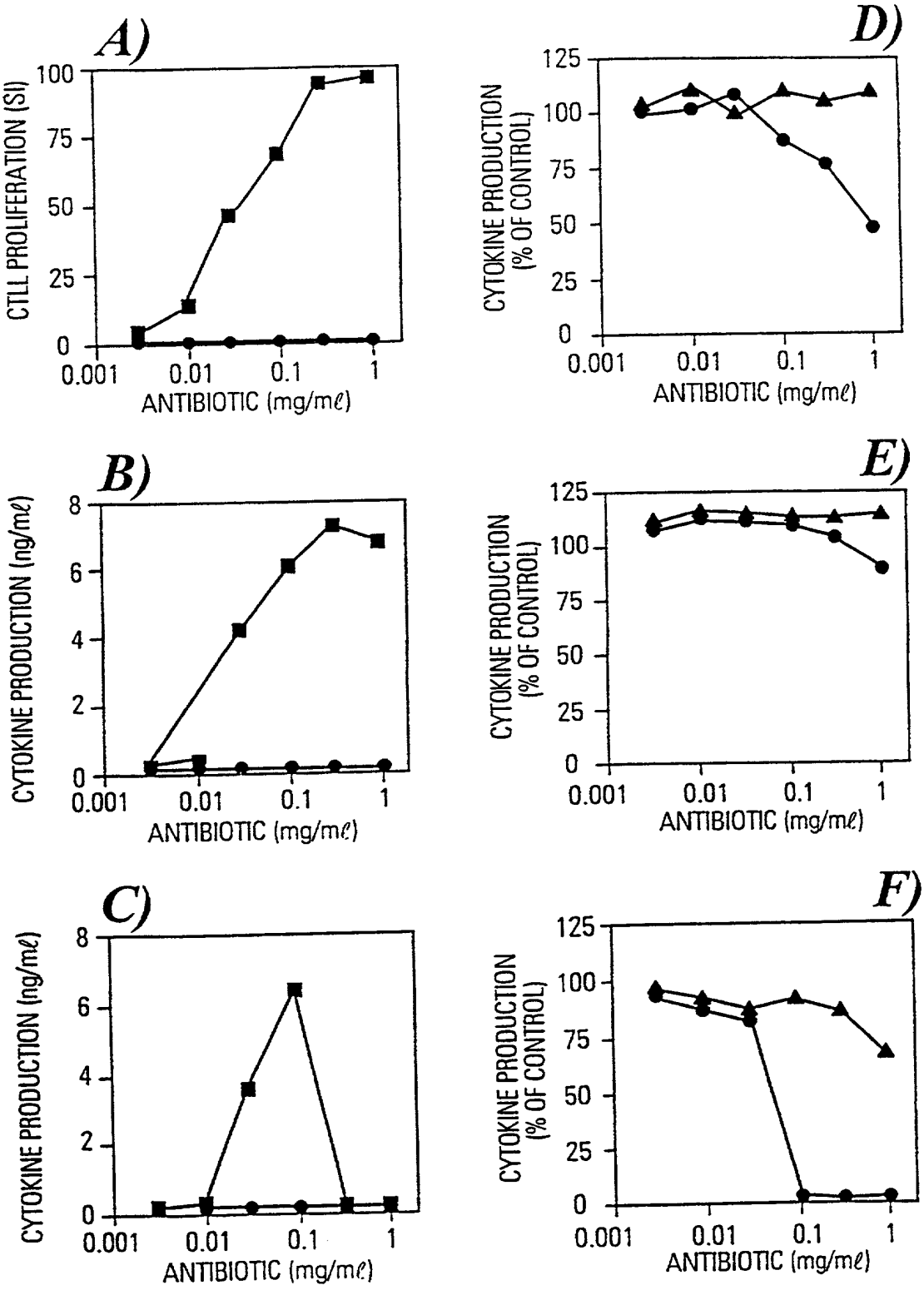
Pg/mℓ
B)
IFNγ
IL4
PHA(μg/mℓ)
Pg/mℓ
C)
TT616-630(μg/mℓ)
Pg/mℓ
D)
PHA(μg/mℓ)
Pg/mℓ

FIG.2

FIG.3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50
25
0
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0.1
1
ANTIBIOTIC (mg/mℓ)
D)
CYTOKINE PRODUCTION (% OF CONTROL)
125
100
75
50
25
0
0.001
0.01
0.1
1
ANTIBIOTIC (mg/mℓ)
B)
CYTOKINE PRODUCTION (ng/mℓ)
8
6
4
2
0
0.001
0.01
0.1
1
ANTIBIOTIC (mg/mℓ)
E)
CYTOKINE PRODUCTION (% OF CONTROL)
125
100
75
50
25
0
0.001
0.01
0.1
1
ANTIBIOTIC (mg/mℓ)
C)
CYTOKINE PRODUCTION (ng/mℓ)
8
6
4
2
0
0.001
0.01
0.1
1
ANTIBIOTIC (mg/mℓ)
F)
CYTOKINE PRODUCTION (% OF CONTROL)
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50
25
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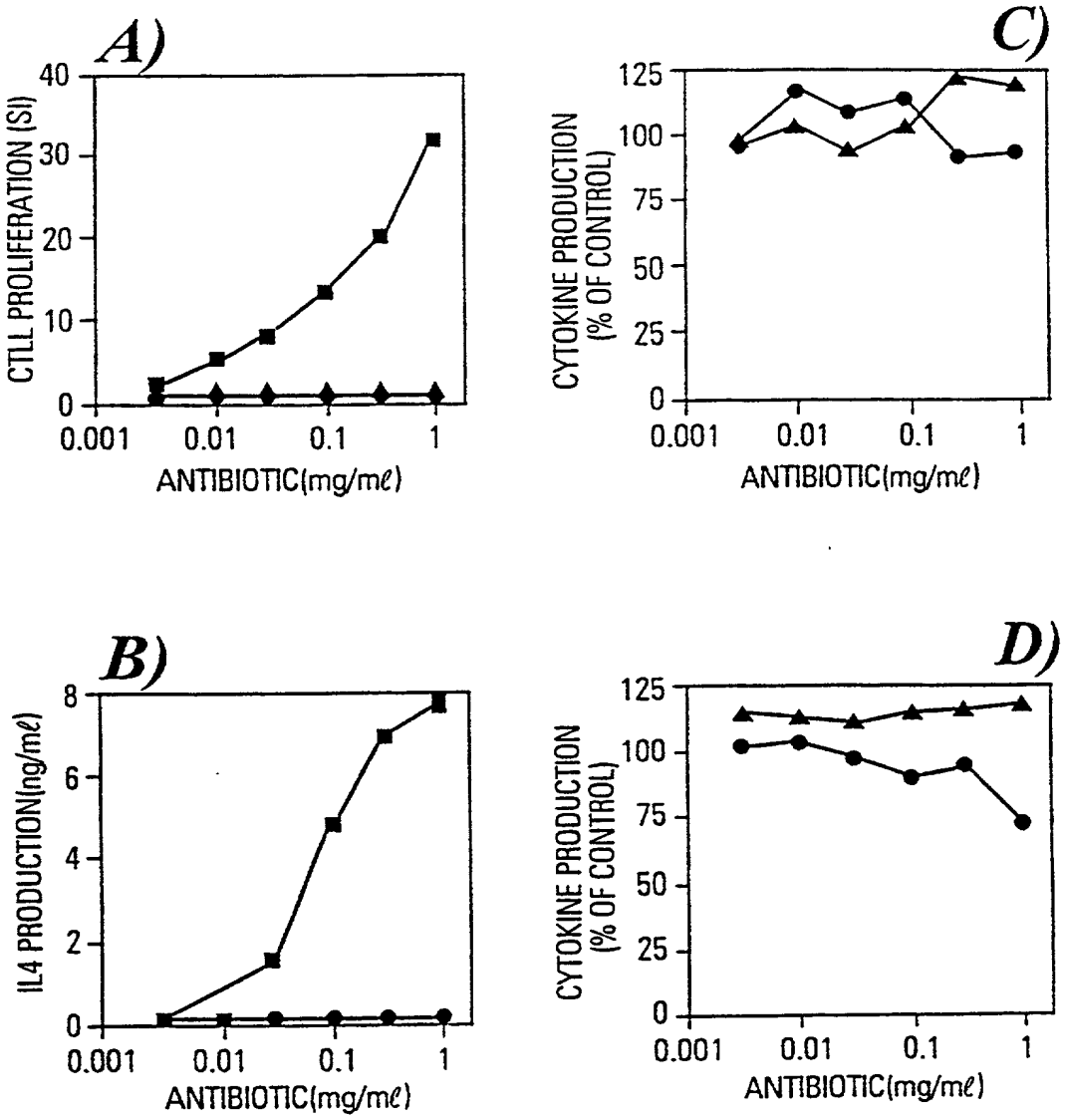
0.001
0.01
0.1
1
ANTIBIOTIC (mg/mℓ)

FIG.4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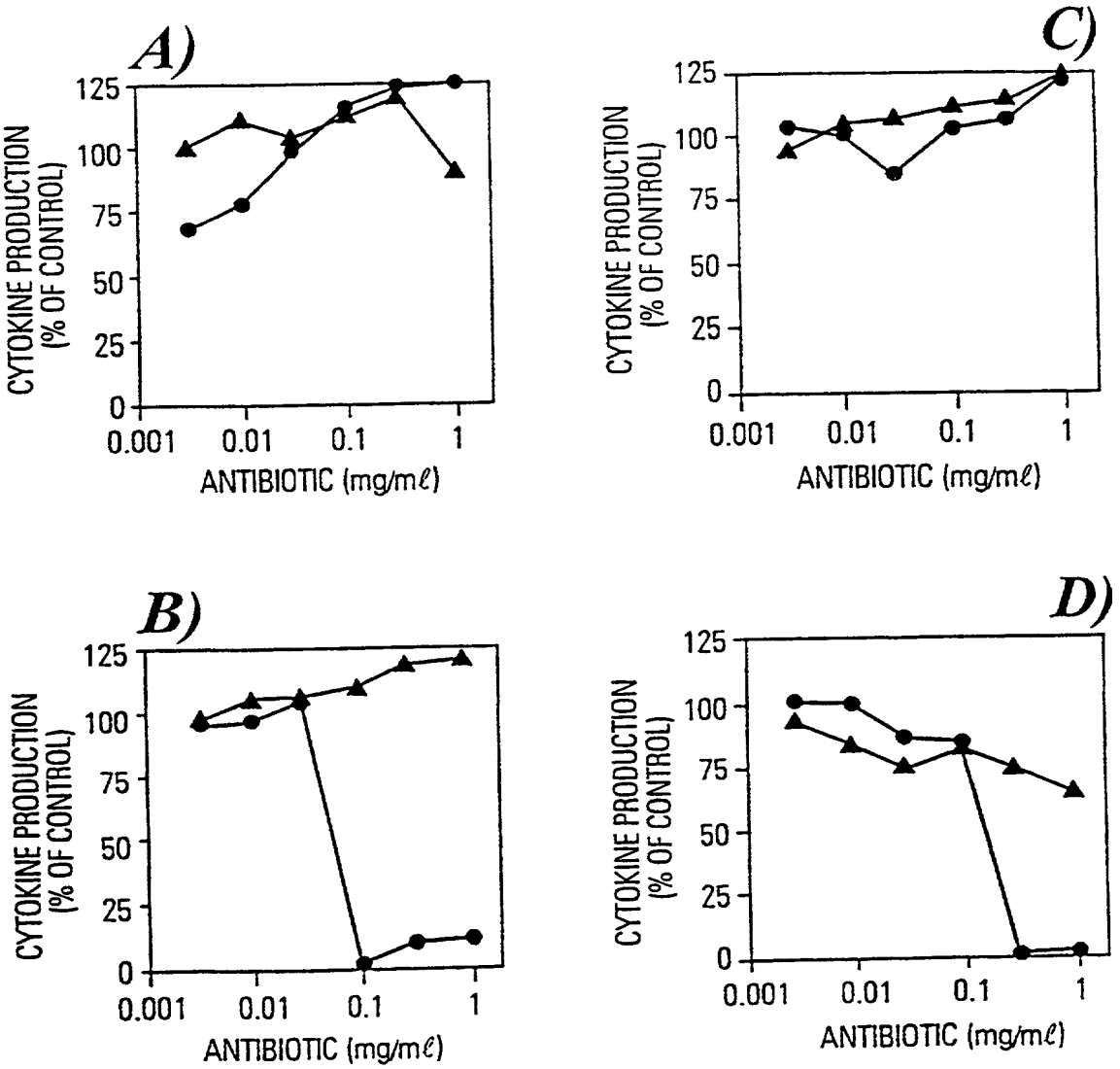
ANTIBIOTIC(mg/mℓ)
C)
CYTOKINE PRODUCTION (% OF CONTROL)
125
100
75
50
25
0
0.001
0.01
0.1
1
ANTIBIOTIC(mg/mℓ)
B)
IL4 PRODUCTION(ng/mℓ)
8
6
4
2
0
0.001
0.01
0.1
1
ANTIBIOTIC(mg/mℓ)
D)
CYTOKINE PRODUCTION (% OF CONTROL)
125
100
75
50
25
0
0.001
0.01
0.1
1
ANTIBIOTIC(mg/mℓ)

FIG.5


A)
C)
B)
D)
CYTOKINE PRODUCTION (% OF CONTROL)
125
100
75
50
25
0
0.001
0.01
0.1
1
ANTIBIOTIC (mg/mℓ)

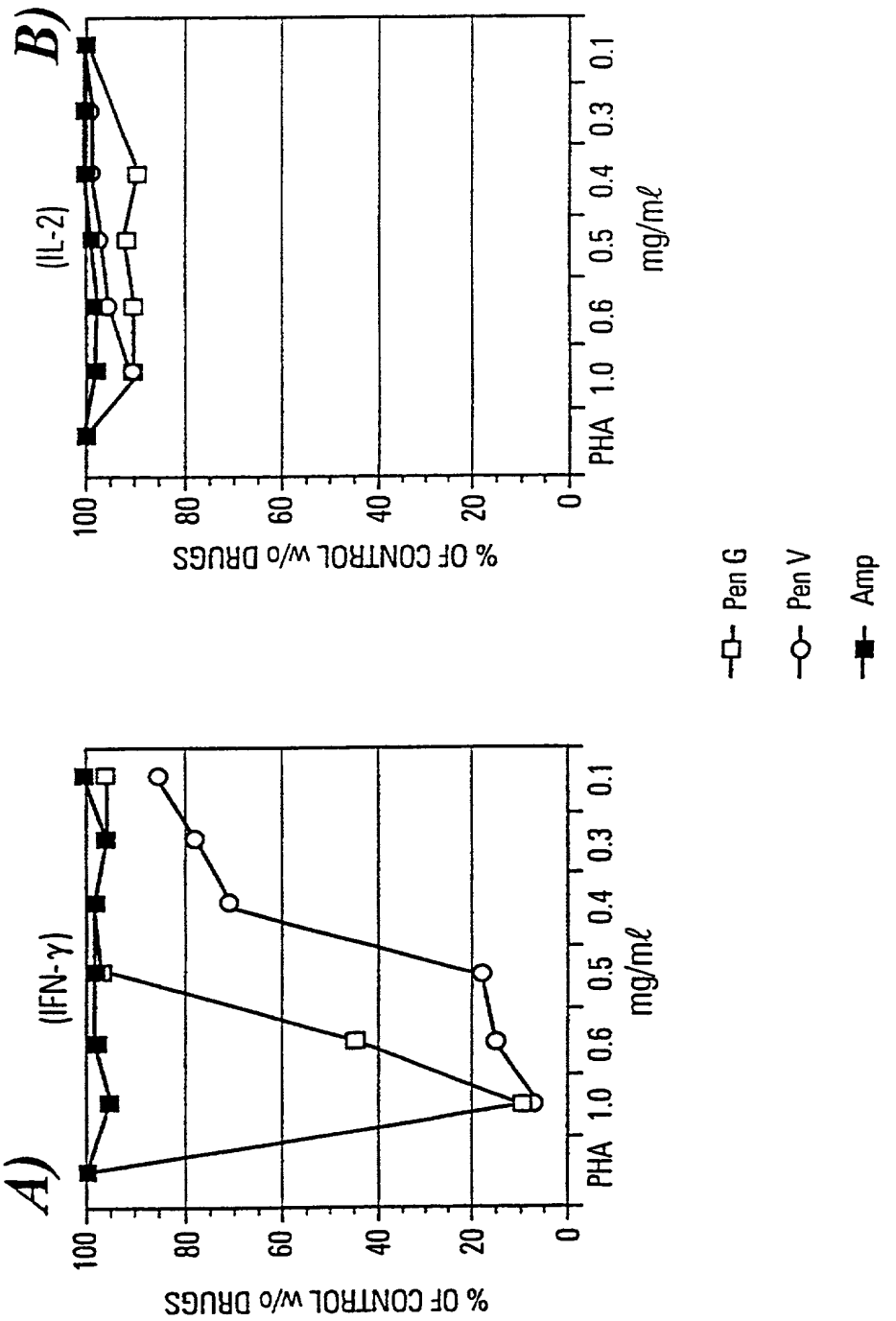

A)
(IFN-γ)
B)
(IL-2)
% OF CONTROL w/o DRUGS
0
20
40
60
80
100
PHA 1.0 0.6 0.5 0.4 0.3 0.1
mg/mℓ
Pen G
Pen V
Amp

FIG.6

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My resident, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "Penicillins as pharmaceuticals for the downregulation of IFNγ production", the specification of which

( ) is attached hereto.

(x) was filed on 23 Dec 1999 as International Application Serial No. PCT/EP99/10378

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

Foreign Priority Applications

I hereby claim foreign priority benefits under Title 35, United States Code 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Priority Claimed

(Number)	(Country)	(Day/Month/Year Filed)	
98 124 591.3	Europe	23 Dec 1998	Yes (xx) No ( )
			Yes ( ) No ( )

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)

U.S. Priority Applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Applic. Serial No.)	(Filing Date)	(Status-patented/pending/abandoned)
(Applic. Serial No.)	(Filing Date)	(Status-patented/pending/abandoned)

Power of Attorney

I hereby appoint the following attorneys and patent agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Peter F. Felfe, Reg. No. 20,297; John E. Lynch, Reg. No. 20,940; Norman D. Hanson, Reg. No. 30,946; John A. Bauer, Reg. No. 32,554; Mary Anne Schofield, Reg. No. 36,669; James Zubok, Reg. No. 38,671; James R. Crawford, Reg. No. 39,155, Andrew Im, Reg. No. 40,657 and David Rubin, Reg. No. 40,314; my attorneys with full power of substitution and revocation.

Address all telephone calls to _____ at (212) 318-3000.

Address all correspondence to: FULBRIGHT & JAWORSKI L.L.P.
666 Fifth Avenue
New York, New York 10103

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(1) Elisabetta Padovan — Signature — 12.06.01
Full Name/Sole or First Inventor — Signature — Date

Residence: 4057 Basel
Switzerland CHX

Post Office Address: ~~Hammerstraße 115~~ LEUENGASSE, 24
4057 Basel, Switzerland

Citizenship: Italian

12.11.'01

(2) Hans Ulrich Weltzien — Signature — 25.6.01
Full Name/Second Inventor — Signature — Date

Residence: 79110 Freiburg
Germany DEX

Post Office Address: Schillhof 5
79110 Freiburg, Germany

Citizenship: German

United States Patent & Trademark Office

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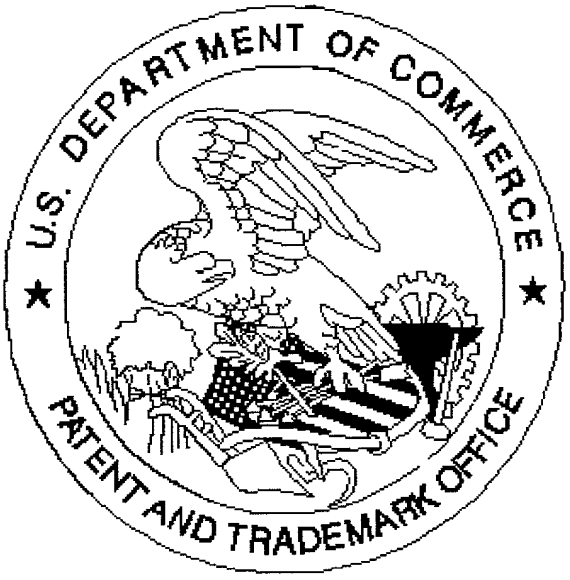

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